Biochimica et Biophysica Acta, 1066 (1991) 70–76

ADONIS 000527369100264M

BBAMEM 75290

Three regions of erythrocyte band 3 protein are phosphorylated on tyrosines: characterization of the phosphorylation sites by solid phase sequencing combined with capillary electrophoresis

Drakoulis Yannoukakos [1], Helmut E. Meyer [2], Corinne Vasseur [1], Catherine Driancourt [1], Henri Wajcman [1] and Elizabeth Bursaux [1]

[1] *INSERM U299, Hôpital de Bicêtre, Kremlin-Bicêtre (France) and* [2] *Institut für Physiologische Chemie, Abteilung für Supramolekulare Systeme, Ruhr-Universität Bochum, Bochum (Germany)*

(Received 30 January 1991)

Key words: Band 3 protein; Phosphorylation; Phosphotyrosine; Capillary electrophoresis; (Human erythrocyte membrane)

The major part of band 3 phosphorylation was recently shown to concern the first tryptic peptide of the protein (Yannoukakos et al. (1991) Biochim. Biophys. Acta 1061, 253–266). Tyrosine 8 is the prevalent site of phosphorylation, but other phosphorylated regions were found which could not be analyzed with certainty. Direct characterization of the phosphorylated residues in all these phosphorylated fragments was made possible due to recent advances in protein chemistry techniques, such as solid phase sequence analysis and capillary electrophoresis. The present report establishes that band 3 phosphorylation occurs predominantly on tyrosines: besides tyrosine 8 already known in the N-terminal region, two other tyrosines are demonstrated to be targets for the tyrosine kinase, tyrosine 359 and tyrosine 904. These residues lie in regions of band 3 exposed to the cytoplasm, the junction of the cytoplasmic and the membrane-spanning domains, and the C-terminal end of the protein which is also cytosolic, respectively.

Introduction

Phosphorylation of erythrocyte membrane proteins has been a subject of considerable interest for the last years as this mechanism has been shown to regulate the associations of several skeletal proteins [1]. The main integral protein, band 3, is also phosphorylated but the physiological role of this reaction is still unclear.

Band 3 phosphorylation depends on the action of at least three kinases: two caseine-kinases, a membrane-bound and a cytosolic one, and a specific band 3 tyrosine kinase [2]. Both caseine kinases phosphorylate preferentially on serine and threonine residues, whereas the tyrosine kinase phosphorylates only tyrosine residues. Compared to the phosphorylation of other erythrocyte membrane proteins, phosphorylation on tyrosine residues appears very specific to band 3 [3].

It has recently been shown that the major part of band 3 phosphorylation concerns the first tryptic peptide of the protein (sequence 1–56) [4]. The tyrosine residue at position 8 represents the prevalent site of phosphorylation, but other phosphorylated residues were found: tyrosines 21 and 46, serines 29 and 50 and some threonine residues located at the end of this peptide. In addition two other regions of band 3 were found phosphorylated, tentatively assigned to: (i) the peptide at the junction of the cytoplasmic and the transmembrane domains of the protein (residues 347–360), and (ii) the C-terminal tryptic peptide (residues 893–911).

Direct characterization of the phosphorylated residues in all these phosphorylated fragments was made possible due to recent advances in protein chemistry techniques, such as solid phase sequence analysis and capillary electrophoresis. The present report establishes that band 3 phosphorylation occurs predominantly on tyrosines: besides tyrosine 8 already known in the N-terminal region, two other tyrosines are demonstrated to be targets for the tyrosine kinase, tyrosine 359 at the junction of the cytoplasmic and the membrane-spanning domains and tyrosine 904 at the C-terminal end of the protein which is also cytosolic [5].

Correspondence: E. Bursaux, INSERM U299, Hôpital de Bicêtre, 94275 le Kremlin-Bicêtre, France.

Materials and Methods

Blood samples were taken from healthy volunteers. The red blood cells were isolated and washed in phosphate-buffered saline. Red cell membranes were prepared by hypotonic hemolysis as previously described [4].

Phosphorylation reaction

The phosphorylation reaction was performed in a 30 mM Hepes buffer (pH 7.0), by incubating the membranes for 10 min at 30°C with [γ-^{32}P]ATP 0.01 mM (37 GBq/mmol) (Amersham), 10 mM Mg^{2+}, in the presence of 0.03 mM orthovanadate. At the end of the phosphorylation reaction the sample was placed on ice and diluted with six volumes of ice cold buffer without [γ-^{32}P]ATP, and containing 0.03 mM orthovanadate, 15 mM p-NPP and 10 mM NaF to inhibit the phosphatases. Thereafter, careful attention was paid to inhibit the phosphatases throughout all the procedures, even during the tryptic digestion of the purified polypeptides. The presence of vanadate is mandatory to inhibit the very active tyrosine phosphatase which copurifies with band 3 protein [4]. In addition, the use of membranes and not whole cells, the low concentration of ATP (0.01 mM), the short phosphorylation time, and the use of a potent tyrosine phosphatase inhibitor, all favor the selection of phosphorylation on tyrosine residues.

Purification of band 3 and its proteolytic fragments

Band 3 protein and its two proteolytic fragments, namely the cytoplasmic and membrane spanning domains, were prepared from these phosphorylated membranes.

Band 3 protein was extracted from the membranes by Triton X-100 (0.5%), and purified by ion-exchange chromatography followed by thiol-exchange affinity chromatography in the presence of Triton X-100 (0.5%) [6]. The cytoplasmic domain of band 3 was cleaved by α-chymotrypsin on inside-out vesicles and isolated by DE 52 cellulose chromatography as described previously [7]. The isolated fragment was deionised and freeze-dried. The membrane-spanning domain was isolated after tryptic cleavage of the cytoplasmic domain directly on ghosts. It was purified from a Triton extract of skeletal proteins-depleted membranes by ion exchange chromatography as described in [4].

Peptide isolation by RP-HPLC

For peptide purification, isolated cytoplasmic fragment of band 3, dissolved in 50 mM ammonium bicarbonate buffer (pH 8.8), was digested at room temperature for 18 h with trypsin (TPCK treated, Merck, Darmstadt, F.R.G.) using an enzyme to the substrate ratio of 3% (w/w). Whole band 3 and its membrane-spanning domain were also digested by trypsin, but the hydrolysis was carried out in the elution buffers used in chromatographic isolation, adjusted to pH 8.8. The peptides were separated by RP-HPLC using a C8 column (Aquapore RP-300, Brownlee Labs, Santa Clara, CA). Peptides were eluted with a flow rate of 1 ml/min, using a 0 to 75% acetonitrile gradient in 0.05% trifluoroacetic acid (TFA) in water. The N-terminal tryptic peptides, isolated by RP-HPLC, were further cleaved by cyanogen bromide as previously described [6]. The ^{32}P-labeled peptides (peaks A, B, C, and D) were isolated from the tryptic digests of the cytoplasmic and membrane spanning fragments. Analysis of the tryptic peptides of whole band 3 protein allowed an estimation of the relative intensities of these four peaks.

Solid phase sequencing

A suitable solid phase support, allowing a highly efficient covalent attachment of the phosphotyrosine-containing peptide was used [8]. 100–300 pmoles of the peptides were dissolved in 20 μl of 30% aqueous acetonitrile and applied onto a Sequelon-AA disk placed on a piece of aluminum-foil. The disk was allowed to dry thoroughly at room temperature. Thereafter, 10 μl of a freshly prepared solution containing 0.1 M 4-morpholinoethanesulfonic acid (pH 5.0), 15% acetonitrile and 10 mg 1-ethyl-3-(dimethylaminopropyl)carbodiimide per ml were carefully applied to the disk. The reaction was allowed to proceed for 20 min at room temperature.

Sequence analysis was performed using the Applied Biosystems model 470 gas-phase sequenator connected on-line to a model 120 PTH-analyzer. The peptide containing Sequelon-AA disk was placed in the reaction cartridge as usual and covered with a TFA-pretreated glass-fiber disk to enhance the performance of the degradation reactions. ATZ (aminothiazolinone)-phosphotyrosine is just insoluble in butyl chloride (the usually employed extraction solvent in the sequenator) but soluble in TFA. Therefore butyl chloride was exchanged for neat TFA for the splitting and transfer of the ATZ derivatives [9].

Following Edman-degradation, half of the resulting phenylthiohydantoin amino acids (PTH-amino acids) was directed toward the on-line PTH-analyzer. The second half of the PTH amino acid sample of each degradation step was transferred into 500 μl Eppendorf vials, dried under vacuum in a Speed Vac and dissolved in 5 μl of 20% aqueous acetonitrile containing 0.35% TFA and 50 mM NaCl as sample solution.

Determination of PTH-phosphotyrosine by capillary electrophoresis

PTH-phosphotyrosine was strictly identified by capillary electrophoresis. A model 270A from Applied Biosystems was used. The capillary length was 72 cm

(50 cm to the detector) and its internal diameter was 50 μm. A 25 kV negative polarity was applied in order to allow the migration of PTH-phosphoamino acids. Electrophoresis was performed in 20 mM sodium citrate buffer (pH 2.5, at 30°C). PTH-amino acids were detected at 261 nm, with a full scale deflection of 0.008 A.U.

Partial hydrolysis of phosphoamino acids containing peptides and derivatisation of liberated amino acids to their PTH derivatives

100–500 pmoles of peptides were partially hydrolysed with 6 M HCl in the presence of phenol for 2 h at 110°C. Capillary electrophoresis analysis was performed after conversion of the amino acids into PTH derivatives, according to Meyer et al. [9].

Results

As previously described, the elution pattern of the tryptic digest of whole band 3 isolated from membranes phosphorylated with [γ-^{32}P]ATP displays four radioactive peaks (Fig. 1) . It should be pointed out that all the radioactivity was eluted with the peptides, without any radioactive peak in front of the chromatography. The relative amounts of labeling was 8, 12, 60, 20% for peaks A, B, C, and D, respectively. Despite quite different preparation procedures of band 3 and its proteolytic fragments, the relative ratio of radioactivity in peaks A, C, and D in the cytoplasmic fragment was the same as that measured on the whole band 3 protein. Similarly, the relative ratio of radioactivity in peaks A and B in the membrane spanning fragment

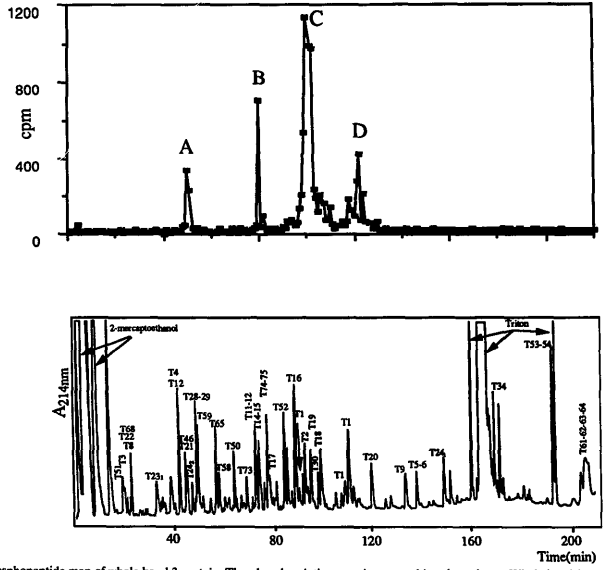

Fig. 1. Phosphopeptide map of whole band 3 protein. The phosphorylation reaction was achieved on ghosts. Whole band 3 protein was thereafter isolated as described in Methods. It was digested with trypsin in the buffer used for separation adjusted to pH 8.8, still containing Triton X-100 and β-mercaptoethanol, in the presence of phosphatase inhibitors (0.03 mM orthovanadate, 15 mM p-NPP, and 10 mM NaF). One mg tryptic digest was applied onto the C8 column for RP-HPLC. Elution was performed at a flow rate of 1 ml/min, using the following gradient between solvent A (TFA 0.05% in water) and solvent B (TFA 0.2% in water/acetonitrile: 25:75): 0 to 30% solvent B from 0 to 90 min, 30 to 40% solvent B from 90 to 140 min, 40 to 47% solvent B from 140 to 155 min, 47 to 67% solvent B from 155 to 170 min, and 67 to 100% solvent B from 170 to 220 min. Lower panel: absorbance profile at 214 nm. Upper panel: radioactive profile of the same digested sample.

was the same as that measured on the whole band 3 protein.

Peak A and peak B, containing very little peptide material, could not be fully characterized. Peaks C and D were identified as the N-terminal tryptic peptide T1 [4].

Phosphorylation of the C-terminal peptide

Radioactive peak B corresponded to a small absorbance peak eluting 5 min before the C-terminal tryptic peptide T74–75 during RP-HPLC analysis. It was observed in the whole band 3 digest as well as in its membrane spanning fragment and was tentatively ascribed to a phosphorylated species of this C-terminal peptide of the protein [4].

After phosphorylation with labeled ATP, this peak was pooled from chromatography of 2 mg digest and its sequence determined. The results showed that this peak contained peptide T74–75, contaminated by a small amount of fragments derivated from T52 and T30. P-Tyr was identified by capillary electrophoresis in the 12th cycle with a decreasing overlap in the following steps (Fig. 2). This result was confirmed by radioactive counting (Fig. 3). Consequently tyrosine 904 was identified as the phosphorylation site in this peptide.

Phosphorylation of peptide T28–29

Peak A was found in the peptide map of the cytoplasmic domain. It was assigned to peptide T28–29, at the C-terminal end of this fragment which results from the chymotryptic cleavage, on inside-out vesicles, of band 3 protein at phenylalanine 379.

A radioactive peak, with the same retention time, was also found in the peptide profile of the membrane spanning fragment. This peak could not be directly assigned to peptide T28–29 as this peptide is not expected in this fragment, the tryptic cleavage of band 3 protein on ghosts taking place at lysine 360.

Sequencing of these two radioactive peaks gave identical results, so that radioactive peak A found in the membrane spanning domain was identified to the phosphorylated tryptic peptide T28–29. P-Tyr was characterized by capillary electrophoresis and radioactive counting in the 13th cycle with a decreasing overlap in the following steps, indicating that tyrosine 359 was the site of phosphorylation (Fig. 3).

It appears therefore that tyrosine 359 phosphorylation inhibits the tryptic cleavage at lysine 360 obtained when band 3 is digested on ghosts under the mild conditions used to generate the membrane spanning fragment (1 h at 0°C). The cleavage occurs then at arginine 346 and the T28–29 is included in this fragment. This explains why peptide T28–29 was found in the tryptic digest of the membrane spanning fragment only when phosphorylated. The peptide bond at lysine

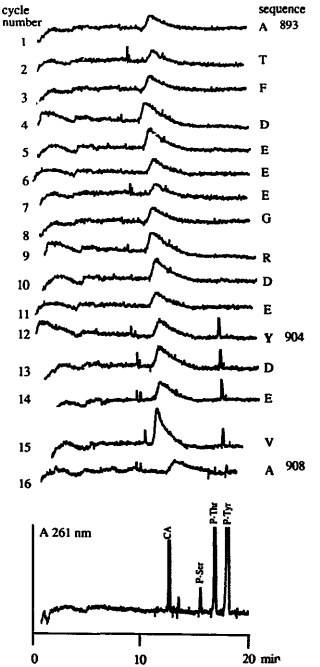

Fig. 2. Capillary electrophoresis of PTH-amino acids of the C-terminal peptide. The C-terminal tryptic peptide of band 3 was isolated by RP-HPLC and submitted to sequence analysis. 50% of each degradation step was collected, dried under vacuum, dissolved in 20 mM sodium citrate buffer (pH 2.5), 20% aqueous acetonitrile, 0.35% TFA, 50 mM NaCl, and analyzed by capillary electrophoresis in 20 mM sodium citrate buffer (pH 2.5) (for details see Methods). Phosphotyrosine appears on the 12th cycle, with overlay in the succeeding steps. The absorbance pattern of the standards (CA = cysteic acid) is shown at the bottom of the graph.

360 is nevertheless cleaved by trypsin during complete digestion of the isolated membrane spanning fragment (18 h at room temperature).

Phosphorylation of the N-terminal peptide

Peaks C and D both correspond to the N-terminal T1 peptide of the protein (sequence 1–56) [4]. Peaks C and D were partially hydrolyzed by 6 M HCl for 2 h at 110°C and the resulting amino acids were transformed

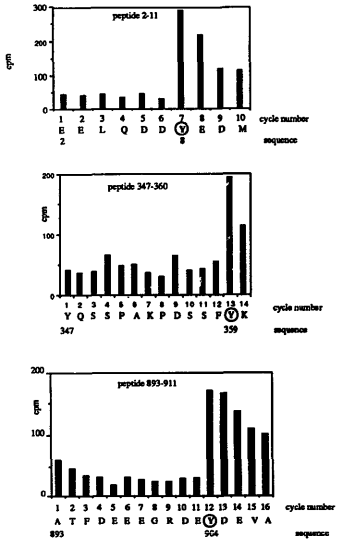

Fig. 3. Radioactive counting of PTH-phosphoamino acids of peptides T1 (2–11), T28–29 (347–360), and T74–75 (893–911). Peptide 2–11 was isolated by RP-HPLC after CNBr cleavage of peptide T1. Peptide T28–29 was isolated by RP-HPLC from the tryptic digests of both the cytoplasmic domain and the membrane spanning domain. Peptide T74–75 was isolated from the tryptic digest of the membrane spanning domain. Each peptide was submitted to sequence analysis and each degradation step analyzed for radioactivity.

to PTH-AA. Phosphoamino acids were then analyzed by capillary electrophoresis. P-Tyr was identified in the two forms of peptide T1 with the following proportions C/D: 5.4:2.3. Trace amounts of P-Thr were found as expected only in peak D but could not be accurately quantified as PTH-phosphothreonine is not very stable. This method did not allow the determination of PTH-phosphoserine which is even less stable, and is almost completely destroyed during the hydrolysis and derivatization steps. Anyhow, transformation of P-Ser in phenylthiocarbamyl-*S*-ethylcysteine, a specific method for measuring P-Ser [10], revealed only trace amounts of this derivative (results not shown).

Peaks C and D could not be sequenced directly because the N-terminal methionine is acetylated. These two tryptic peptides were therefore further cleaved by cyanogen bromide and sequencing was performed on the resulting fragments (sequences 2–11, 12–31, 13–31, 32–56) of each peak. The presence of a methionine at both positions 11 and 12 is responsible for several possibilities of cleavage and heterogeneity due to oxidation state.

After CNBr cleavage of peak C, radioactivity was found only in fragment 2–11. P-Tyr was identified by capillary electrophoresis in the 7th step with a decreasing overlap in the following steps. Radioactive counting confirmed the above results (Fig. 3). Consequently tyrosine 8 was confirmed as the site of phosphorylation.

After CNBr cleavage of peak D, no radioactivity was present in fragment 2–11 and very little in the fragment 32–56. Most of the labeling was found in the several peaks resulting from sequence 12–31. Phosphorylation of tyrosine 21 was thus indicated as (i) the presence of P-Tyr was demonstrated by capillary electrophoresis in peak D, and (ii) tyrosine 21 is the only tyrosine in the fragment 12–31. Besides tyrosine 21, the radioactivity was shown previously to be distributed to tyrosine 46 (when the phosphorylation reaction was carried out in the presence of Mn^{2+} only) and to serine 29 in fragment 12–31, and to serine 50 and to one or several threonines, in fragment 32–56 [4]. Due to the heterogeneity of these peaks and to the consequently faint radioactive labeling, the capilllary electrophoresis and radioactive counting of successive sequence steps of these two fragments failed to characterize the sites of phosphorylation.

Discussion

The extent of phosphorylation depends on the equilibrium between kinase and phosphatase activities, varying thus with experimental conditions. We recently showed that a phosphotyrosine-phosphatase is copurified with band 3 protein [4]. Its activity is decreased at low temperature (0°C) and completely inhibited by orthovanadate. Phosphoserine and phosphothreonine-phosphatase activities are inhibited by sodium fluoride and low temperature.

Contradictory results have been published concerning the distribution of phosphorylation on band 3 protein, after phosphorylating the ghosts with labeled ATP, in the presence of Mg^{2+}. Dekowski et al., studying the phosphorylation of the 23 kDa N-terminal fragment of band 3, showed that the major phosphoamino acid was phosphotyrosine, and that one site was tyrosine 8 [11]. By contrast Phan Dinh Tuy et al. found only 9% phosphotyrosine among phosphoamino acids (P-Ser and P-Thr) in band 3 [12], while Vasseur et al. [13] found 50% phosphotyrosine. All these results were obtained by electrophoresis after partial hydrochloric acid hydrolysis. This procedure leads to a partial loss of P-Tyr which is more labile in the acid than the other

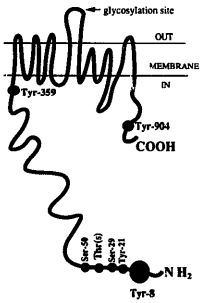

Fig. 4. Scheme of the phosphorylated regions in band 3 protein. Band 3 protein is phosphorylated mainly on tyrosine residues, in 3 regions: the N-terminal, the C-terminal end of the protein and the junction peptide between the cytoplasmic and the membrane spanning domains.

two phosphoamino acids. Our strategy avoided these misleading procedures.

The present results demonstrate that there are at least three dominant sites for tyrosine phosphorylation in band 3 protein: tyrosine 8, tyrosine 359, and tyrosine 904. These three residues account for more than 80% of the total labeling of the protein. The remainder of the radioactivity seems to be evenly distributed among tyrosine 21, serines 29 and 50 and threonine(s) of the region 39–54 (Fig. 4). In any case, the phosphoamino acid distributions in vivo may be vastly different.

Most of the phosphorylation was localized on the N-terminal peptide. Nearly all the residues susceptible to phosphorylation in this region were labeled, but tyrosine 8 was by far the main phosphorylated residue. When this residue was phosphorylated, no other phosphorylation reaction occured within this peptide [4]. Tyrosine phosphorylation occured in two other regions: at the junction of the two domains of the protein, the cytoplasmic and the membrane spanning domains, and in the C-terminal part of the protein, which may be of physiological significance.

These three regions share as a common property to be rich in charged residues. Tyrosine 359 lies among residues which may form a positively charged cluster (between residues 340 and 360 there are four arginines and two lysines). By contrast, tyrosines 8 and 904 are surrounded by acidic residues. The sequence of the C-terminal of the protein is nearly a mirror image of the N-terminal part: acidic residues predominate in both portions in which eight out of fourteen in the latter, and eight out of sixteen in the former, are glutamic acid or aspartic acid, with two acidic residues on each side of the phosphorylated tyrosine in both cases. These sequences are well known recognition sites for tyrosine protein-kinases and their importance has been demonstrated with model peptides in several instances [14]. The phosphorylation site motif of tyrosine 359 is more like those described as targets for caseine kinases. Indeed erythrocyte caseine kinase has been found to catalyse the phosphorylation of band 3 tyrosine [15]. Nevertheless, it has been reported that some tyrosine residues, targets for the erythrocyte tyrosine kinase, are not surrounded by acidic residues [16].

These hydrophilic regions of the protein, rich in acidic or basic residues, are likely to be easily accessible for interactions with cytosolic and skeletal proteins.

The interactions of the N-terminal end have been studied in great detail [17]. Evidence that the hemoglobin tetramer [18] and its oxidized derivatives [19] specifically bind to this area is well documented. The binding of glycolytic enzymes, glyceraldehydephosphate dehydrogenase in particular, was shown to depend on tyrosine 8 phosphorylation [20]. Phosphorylation could modulate these interactions by adding a negative charge, or via structural modifications. A conformational change induced by phosphorylation was already suggested by the shift of hydrophobicity of the N-terminal peptide as shown by RP-HPLC analysis [4]. Preliminary results obtained by circular dichroism of this peptide indicated the possible breaking of an α-helix on tyrosine 8 phosphorylation (manuscript in preparation).

The function of the C-terminal region is less documented. Immunologic studies with monoclonal antibodies allowed its localization on the cytoplasmic side of the red cell membrane [5] which is in agreement with its phosphorylation by membrane-bound kinases. Binding of pyridoxal phosphate in the C-terminal region showed that this region of band 3 constitutes a part of the active center for anion transport [21]. Phosphorylation of tyrosine 904, as that of tyrosine 8 in the N-terminal part of the protein, could induce a conformational modification and thereby modulate anion transport or the interaction of this segment with other cytosolic proteins.

The peptide containing tyrosine 359 connects the two domains of the protein. It is readily accessible to proteases, and mild proteolysis at the cytoplasmic surface causes the release of the entire cytoplasmic domain. This indicates that this portion of the protein is held to the membrane only by the polypeptide backbone, and forms no strong interaction with the membrane spanning domain. It could, on the other hand, interact with the N-terminal part of either the same monomer or of another monomer, leading to the formation of a dimer. Phosphorylation of tyrosine 359, by adding a negative charge to the region, could disrupt

this interaction and play a role in the quaternary structure of the protein.

Among the three phosphorylated tyrosine residues described in human band 3, only tyrosine 904 is conserved in the three species whose band 3 protein primary structure is known, namely human, murine, and chicken. Tyrosine 359 is absent in chicken band 3 but present in the murine band 3. Tyrosine 8 is present only in the human species [22].

No phosphorylation site was localized in the region of the protein supposed to interact with ankyrin (between residues 174 and 317). A participation of the N-terminal of band 3 in this interaction is suggested [23,24]. This could mean that phosphorylation of band 3 plays no role in the interaction with the skeletal proteins, which represents the major function of the cytoplasmic domain. A conformational change induced by phosphorylation could modulate this interaction. Band 3 associates also with band 4.1, in a manner modulated by band 4.1 phosphorylation by protein kinase C [25]. It is still unknown whether band 3 phosphorylation (on any of the described phosphorylation sites) could play a role on its interaction with band 4.1 or band 4.2.

In conclusion, phosphorylation on tyrosines of band 3, the major substrate for erythrocyte tyrosine kinase, occurs on exposed domains of the protein. This topology, on the N- and C- terminal parts, and at the junction of the two domains may have important physiological implications. Modulation of anion self-exchange was shown to depend on ATP at micromolar concentrations [26]. Phosphorylation of band 3 could interfere directly with a part of the protein involved in anion transport, or could act through the modification of the quaternary structure of the protein, as postulated by Salhany [27].

Acknowledgments

We wish to thank Horst Korte for his expert technical assistance. This work beneficited of financial support from the Institut National de la Santé et de la Recherche Médicale, the Fondation pour la Recherche Médicale, the Deutsche Forschungsgemeinschaft, and the Fonds der Chemischen Industrie.

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